





# Clinical Relevance of Type II Fatty Acid Synthesis Bypass in *Staphylococcus aureus*

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**ABSTRACT** The need for new antimicrobials to treat bacterial infections has led to the use of type II fatty acid synthesis (FASII) enzymes as front-line targets. However, recent studies suggest that FASII inhibitors may not work against the opportunist pathogen *Staphylococcus aureus*, as environmental fatty acids favor emergence of multi-anti-FASII resistance. As fatty acids are abundant in the host and one FASII inhibitor, triclosan, is widespread, we investigated whether fatty acid pools impact resistance in clinical and veterinary *S. aureus* isolates. Simple addition of fatty acids to the screening medium led to a 50% increase in triclosan resistance, as tested in 700 isolates. Moreover, nonculturable triclosan-resistant fatty acid auxotrophs, which escape detection under routine conditions, were uncovered in primary patient samples. FASII bypass in selected isolates correlated with polymorphisms in the *acc* and *fabD* loci. We conclude that fatty-acid-dependent strategies to escape FASII inhibition are common among *S. aureus* isolates and correlate with anti-FASII resistance and emergence of nonculturable variants.

**KEYWORDS** antibiotic resistance, infection, persistence, nondetectable resistance, fatty acids, *Staphylococcus aureus*

*Staphylococcus aureus* is a leading cause of a wide range of infections that can affect numerous host organs and cause a range of effects from mild symptoms to severe and life-threatening diseases. Acquisition of antimicrobial resistance, likely due to overuse of antibiotics, is the principal cause of *S. aureus* drug resistance (1). A class of new-generation antimicrobials in intensive development uses the type II fatty acid synthesis (FASII) pathway as an antibacterial target (2–5) to treat *S. aureus* infections (6–8). A widely used biocide, triclosan (5-chloro-2-[2,4-dichlorophenoxy]phenol; commercialized as Irgasan or Microban), is a prototype for further anti-FASII development (4, 9, 10).

The utility of FASII inhibitors was questioned when several Gram-positive bacteria were shown to be refractory to FASII inhibitors in the presence of exogenous fatty acids, making FASII enzymes dispensable (11, 12). Both free and complexed fatty acids are abundant in the host (13, 14), which would facilitate FASII bypass. Reservoirs and invasion sites of clinical and community-acquired staphylococci (i.e., skin, nares, gut, blood, and organs) are naturally rich in fatty acids (13–16), and triclosan is present in the environment and in human body fluids (17, 18). This combination could favor FASII bypass via emergence of triclosan-resistant variants, including fatty acid auxotrophs. Fatty-acid-dependent isolates escape detection on standard isolation media, thereby confounding diagnosis and treatment.

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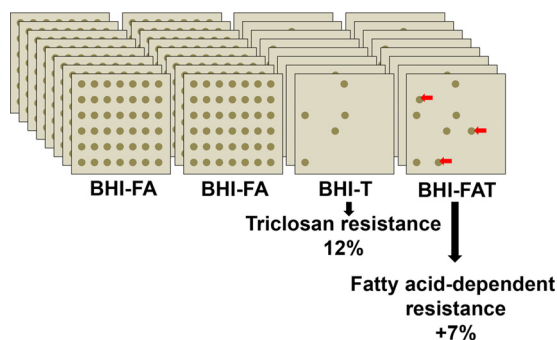
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**FIG 1** Screening strategy to assess fatty-acid-dependent triclosan resistance among clinical and veterinary *S. aureus* isolates (FA-T<sup>r</sup>). Plates contained BHI medium with the indicated fatty acid (FA) and triclosan (T) additives. The fatty acids were C14:0, C16:0, and C18:1 (170  $\mu$ M each); triclosan was used at 0.25  $\mu$ g/ml. Isolates were spotted in the same order on each plate. Those that grew on BHI-FA-T but did not grow, or grew poorly, on BHI-T were scored as FA-T<sup>r</sup> and selected for further study. In total, ~700 isolates were scored using this screen.

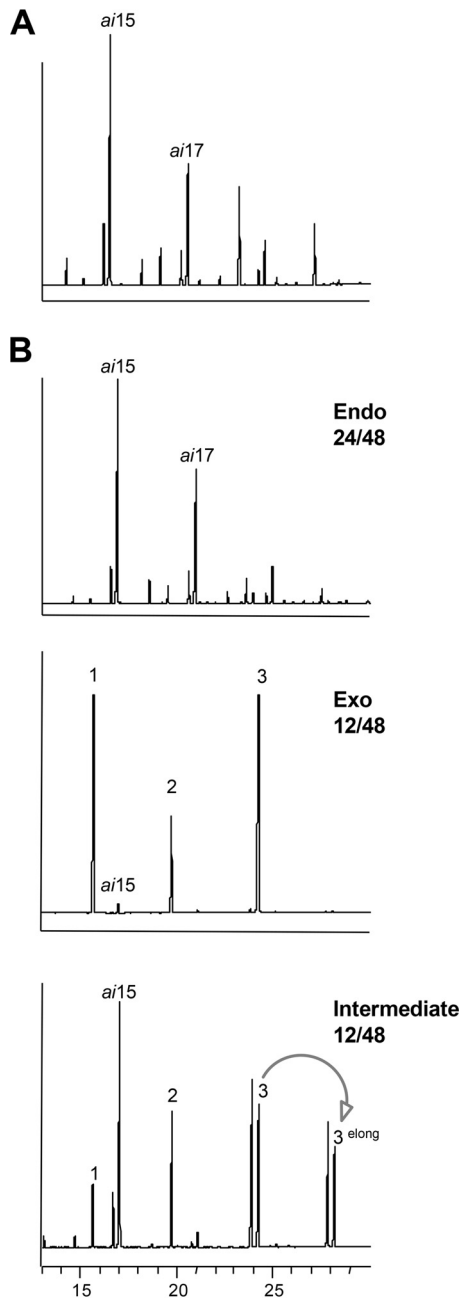
Although *S. aureus* was at first considered sensitive to FASII inhibitors, our recent studies demonstrated benefits from environmental fatty acids for emergence of resistant mutants (19). The question of the survival of such mutants in a clinical context remains in debate (6, 7, 12, 19). It is therefore essential to determine whether FASII bypass, as characterized mainly *in vitro*, is relevant to natural staphylococcal populations.

In this work, we examined a large panel of clinical and veterinary *S. aureus* isolates for the extent of fatty acid impact on triclosan resistance and culturability. Our results demonstrate that isolates using fatty-acid-dependent strategies to escape FASII inhibition are common. Fatty-acid-containing medium gives a more accurate assessment of triclosan resistance in a clinical setting and facilitates identification of pathogens that escape detection by conventional approaches.

## RESULTS

**Fatty acid supplementation reveals a frequent class of triclosan-resistant *S. aureus* among clinical and veterinary isolates.** A four-plate screen using media with or without fatty acids (brain heart infusion [BHI], BHI-triclosan [BHI-T], BHI-fatty acids [BHI-FA], and BHI-fatty acids-triclosan [BHI-FA-T]) (Fig. 1) was set up to search for *S. aureus* isolates that require fatty acids for triclosan resistance. The fatty acids C14:0, C16:0, and C18:1 were chosen mainly as being predominant in two representative environments of localized and systemic infections, skin and serum (20, 21). Human clinical and veterinary mastitis isolates were scored for triclosan resistance (T<sup>r</sup>) and fatty-acid-dependent triclosan resistance (FA-T<sup>r</sup>). Eighty-five (12%) of the 695 tested isolates were scored as triclosan resistant (with growth on BHI, BHI-T, and BHI-FA-T plates), consistent with previous reports of high proportions of triclosan-resistant staphylococci among hospital strains (22, 23). Remarkably, the screen identified 49 isolates (7%) for which triclosan resistance was revealed or markedly enhanced on BHI-FA-T compared to BHI-T (scored as FA-T<sup>r</sup>), raising the total proportion of triclosan-resistant isolates to 19%, i.e., a 58% increase. FA-T<sup>r</sup> isolates were from diverse clinical or veterinary origins, and no overall correlation was observed between the FA-T<sup>r</sup> phenotype and sample sources (see Table S2 in the supplemental material). *spa* typing was performed on FA-T<sup>r</sup> *S. aureus* isolates to confirm their identification and determine whether they occurred within restricted *S. aureus* subgroups (see Table S3 in the supplemental material). FA-T<sup>r</sup> isolates were distributed among various clonal and *spa* types, including the common multilocus sequence typing (MLST) clonal types (CC5, CC8, and CC45) (24). FA-T<sup>r</sup> clones were also present in the emerging clonal type CC398 (25) and in less common clonal types. Broader screening studies will be needed to determine whether FA-T<sup>r</sup> clones preferentially occur in any clonal groups.

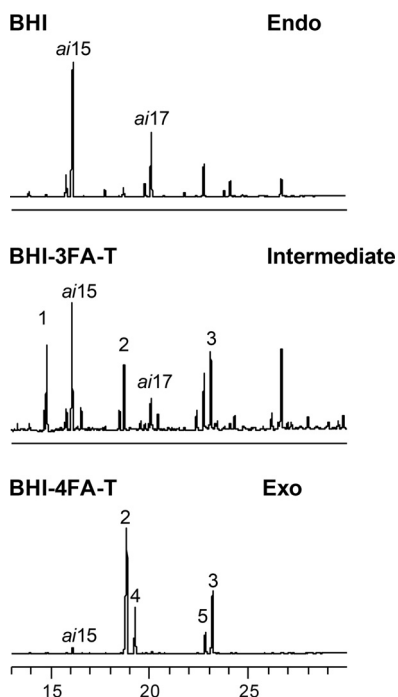
These results establish the frequent existence of clinical and veterinary staphylococci whose triclosan resistance requires the presence of exogenous fatty acids. They



**FIG 2** Three types of FA-T<sup>r</sup> *S. aureus* isolates. (A) Representative fatty acid profile of *S. aureus* isolates grown on nonselective BHI liquid medium. (B) Fatty acid profiles of exogenous (Exo), endogenous (Endo), and combined Exo and Endo (intermediate) fatty acid profiles of FA-T<sup>r</sup> isolates grown in the presence of 3 fatty acids and triclosan (see the legend Fig. 1). The major endogenous fatty acids, anteiso *ai*15 and *ai*17, are indicated. Exogenous fatty acids in medium are as follows: 1, C14:0; 2, C16:0; and 3, C18:1. The arrow indicates elongation via FASII (in this case, C18:0 [endogenous] and C18:1 [3<sup>elong</sup>] are extended to C20:0 and C<sub>20:1</sub>), which suggests that triclosan is not fully inhibitory as tested; in some cases, higher triclosan concentrations shifted profiles to fully exogenous. Note that profiles scored as Exo contain fewer than 2% anteiso forms; above this threshold, they were scored as intermediate.

show that triclosan resistance among medically relevant strains is underestimated due to inappropriate screening conditions.

**Three types of fatty-acid-dependent triclosan resistance.** *S. aureus* strains grown in nonselective BHI medium synthesize mainly branched-chain fatty acids, comprising *ai*15 and *ai*17 (Fig. 2A). In contrast, primary cultures of 48 FA-T<sup>r</sup> isolates grown in BHI-FA-T revealed three types of fatty acid profiles (Fig. 2B): endogenous fatty acids



**FIG 3** Triclosan resistance varies according to the nature of available exogenous fatty acids and their incorporation. Sputum isolate Trsa 180 was initially scored as triclosan sensitive using the four-plate test. The isolate was resuspended in 0.9% NaCl directly from the BHI agar plate and retested for growth on BHI and BHI-FA-T plates containing either the 3 fatty acids (3FA) as used for Fig. 2 or 4 fatty acids (4FA) comprising sputum fatty acids in physiological proportions (see below). Growth was confluent on 4FA-triclosan solid medium, while growth on 3FA-triclosan was poor. Colonies were recovered directly from plates for fatty acid profile determinations. The major endogenous fatty acids, *ai15* and *ai17* are indicated. Exogenous fatty acids in the medium are as follows: 1, C14:0; 2, C16:0; 3, C18:1; 4, C16:1; and 5, C18:0. The global fatty acid concentration was 500  $\mu$ M. For 3FA, the amounts of the fatty acids were equimolar. For 4FA, proportions were 69, 3, 14, and 13%, respectively, for C16:0, C16:1, C18:0, and C18:1.

(Endo) (24 isolates), strictly exogenous fatty acids (Exo) (12 isolates), and mixed profiles (intermediate) (12 isolates). In general, Exo isolates grew more slowly than intermediate or Endo isolates in BHI-FA-T medium (the respective optical density at 600 nm [OD<sub>600</sub>] ranges were 3 to 6, 7 to 11, and 8 to 16 under selective conditions). We noted that FA-T<sup>r</sup> strains having Exo profiles were isolated mainly from two fatty acid-rich niches: human skin and cattle mastitis (Table S2) (20, 26). These observations establish that FASII bypass using available fatty acids is relevant to triclosan resistance in clinical and veterinary isolates.

**Anti-FASII resistance of *S. aureus* isolates varies according to the fatty acid environment.** We asked whether a better match between the fatty acids used in screenings and those from the natural environment would influence triclosan resistance and fatty acid incorporation. For example, an isolate from human sputum (Trsa 180) was initially scored as triclosan sensitive but was triclosan resistant with an Exo profile when grown with fatty acids more closely related to its initial biotope (27) (Fig. 3). Four additional FA-T<sup>r</sup> isolates switched from an intermediate to an Exo profile when grown with fatty acids more closely matching those of their initial biotope. We therefore speculate that the FASII bypass frequencies in natural niches may be greater than estimated in this study.

**Genetic determinants of FA-T<sup>r</sup> resistance.** Half of the FA-T<sup>r</sup> isolates produced endogenous fatty acids despite the presence of triclosan. The well-known FASII routes for triclosan resistance in clinical isolates alter the triclosan target *FabI* via (i) mutations in *fabI* or (ii) the presence of *sh-fabI* (a *fabI* allele that is horizontally transferred from *Staphylococcus haemolyticus*) (10, 22, 28). Among 31 Endo isolates identified in this work, 7 carried *fabI* polymorphisms previously associated with triclosan resistance (see

**TABLE 1** Acc and FabD changes in FA-T<sup>r</sup> isolates that bypass triclosan inhibition by incorporating exogenous fatty acids

Isolate	Relevant AccABCD variation <sup>a</sup>	Relevant FabD variation <sup>a</sup>
Trsa 275	Wild type	Wild type
MT8099	AccC stop aa 445	Wild type
P12	Wild type	FabD <sup>E235Vb</sup>
P1113	Wild type	FabD <sup>G196Dc</sup>
P1127	AccD <sup>D268Nd</sup>	FabD <sup>G196D</sup>
P146	AccD <sup>K35Ne</sup> stop aa 137	FabD <sup>S71Lf</sup>
P176	AccB <sup>K188Eg</sup>	Wild type
MT8348	AccC stop aa 117	Wild type
Trsa 36 <sup>f</sup>	AccD stop aa 111	Wild type
MT8343	Wild type	FabD <sup>L192Ih</sup>

<sup>a</sup>Variants are also present in sequenced genomes reported in public databases (from NCBI, as of 1 January 2015), as indicated for the particular variation. Amino acid differences that were outside conserved regions and present in hundreds of sequenced genomes were not considered.

<sup>b</sup>Thirty-six strains.

<sup>c</sup>FabD variants at position 196 were recently shown to account for FASII bypass (19).

<sup>d</sup>Twenty-two strains.

<sup>e</sup>Fourteen strains.

<sup>f</sup>Thirteen strains.

<sup>g</sup>Eight strains.

<sup>h</sup>Ten strains.

<sup>i</sup>Poor growth on BHI medium.

Table S4 in the supplemental material), and 24 carried the additional *sh-fabI* gene (Table S4). Both mechanisms were previously associated with triclosan resistance not requiring exogenous fatty acids. The mechanism for exogenous fatty-acid-dependent resistance in these Endo isolates remains to be investigated.

Exo isolates (10 tested isolates) carried wild-type *fabI* sequences and lacked secondary *fabI* copies, as expected in a case of FASII bypass. *In vitro*-selected FASII bypass in *S. aureus* was associated with mutations mapping to the *acc* and *fabD* genes (6, 19, 29). Among 10 analyzed Exo isolates, 6 carried polymorphisms in *accC*, *accD*, or *accB*, including stop codons (Table 1). A stop codon in *accD* putatively leads to a truncated protein lacking a domain needed for both enzymatic and regulatory activities (30). Nonauxotrophy of these isolates might be due to supplementary *accBC* copies (which are present in numerous staphylococcal genomes) and/or to partial *accD* read-through (e.g., the *accD* stop in Trsa 36 corresponds to a single-nucleotide deletion in a stretch of six adenines). About half of the Exo isolates studied here carried *fabD* polymorphisms, some of which mapped adjacent to a catalytic residue of the active site (Table 1) (31). Two isolates had the FabD<sup>G196R</sup> mutation (the more common amino acid at position 196 [aa 196] is glycine) that we recently showed to be responsible for FASII bypass (19). In conclusion, the *acc* and/or *fabD* polymorphism identified among FA-T<sup>r</sup> clinical and veterinary isolates is likely responsible for the observed capacity to elude FASII inhibition when fatty acids are available.

**Clinical staphylococci include triclosan-resistant fatty acid auxotrophs.** To search for fatty acid auxotrophs, primary clinical specimens from 103 patients were plated directly on fatty-acid-containing medium that is semiselective for *S. aureus* growth (see Materials and Methods). Colonies were then repatched on BHI, BHI-T, BHI-FA, and BHI-FA-T plates to identify fatty acid auxotrophs. Four fatty acid auxotrophs were recovered in the screen as growing on fatty-acid-containing media (BHI-FA and BHI-FA-T) but not on BHI nor BHI-T (Table 2). Two were identified as *S. aureus* and had Exo profiles even in the absence of triclosan. One *S. aureus* auxotroph was isolated from a bronchial aspirate in which staphylococci were not detected by routine identification. The second *S. aureus* auxotroph was recovered from a burn wound; in this case, the sample contained a mixed population of staphylococci (a lawn and distinct colonies), of which the predominant population (i.e., the lawn) appeared only on fatty-acid-containing media (Fig. 4). Analysis of potential genetic determinants revealed AccB and

**TABLE 2** Fatty acid auxotrophy in hidden clinical isolates

Isolate	Source	Species assignment	Relevant AccABCD variation	Relevant FabD variation	Routine hospital identification
HP6D	Burn	<i>S. aureus</i> CC398	AccC stop aa 94, AccB <sup>A60V,S56N</sup>	FabD <sup>P191T</sup>	<i>S. aureus</i> , <i>S. epidermidis</i>
HP7	Bronchial aspirate	<i>S. aureus</i> CC398	AccC stop aa 94, AccB <sup>A60V,S56N</sup>	Wild type	Streptococci, <i>Neisseria</i>
HP9	Sputum	<i>Staphylococcus hominis</i> (98% 16S rRNA identity)	ND <sup>a</sup>	ND	None
HP8	Bronchoalveolar lavage specimen	<i>Corynebacterium accolens</i> (95% 16S rRNA identity)	ND	ND	<i>S. aureus</i> , <i>Enterobacter cloacae</i>

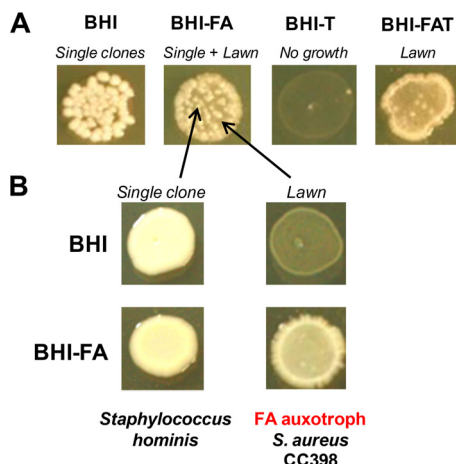
<sup>a</sup>ND, not determined.

AccC variants in both *S. aureus* auxotrophs and a FabD variant in one of them (Table 2). Two non-*S. aureus* fatty acid auxotrophs were also detected: a *Staphylococcus hominis* isolated from sputum and a *Corynebacterium* isolate closely related to the lipid-requiring species *Corynebacterium accolens* (32) (95% 16S rRNA identity) from a bronchoalveolar lavage specimen.

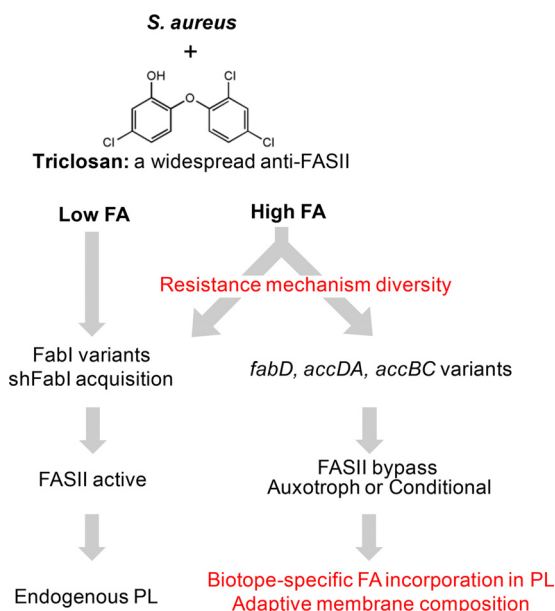
The inclusion of fatty acids in primary screening media thus uncovers a new class of clinical staphylococcal fatty acid auxotrophs that are viable but nonculturable (VBNC). Fatty acid auxotrophs were previously selected in the laboratory using FabI inhibitors in the presence of fatty acids (19, 29). The present results lead us to suggest that triclosan may select for successful FASII bypass variants in the human host.

**DISCUSSION**

Our recent work demonstrated that fatty acids enable *in vitro* emergence of infectious *S. aureus* mutants that are resistant to bacterial FASII inhibitors, including triclosan (19). In this study, we demonstrate that FASII bypass, using environmental fatty acids, is clinically relevant and appears to be a common triclosan resistance strategy in *S. aureus*. We show that fatty-acid-dependent bypass of this FASII inhibitor accounts for resistance among clinical and veterinary isolates, thus attesting to competitiveness and potential success of such strains as pathogens in the host. Our results further indicate that previous determinations of triclosan resistance among clinical strains have been underestimated. The proportion of currently undetected resistance may be even higher, as fatty acid combinations that favor resistance might vary according to the site of infection.



**FIG 4** A hidden FA-T<sup>r</sup> *S. aureus* fatty acid auxotroph in a burn patient. Primary clinical samples were plated directly on a semiselective plate (HiChrome agar), to which oleic acid (C18:1) (500 μM) was added. (A) The FA-T<sup>r</sup> isolate was then identified using the four-plate screen (for the method, see Fig. 1). (B) The isolate comprises a *S. aureus* fatty acid auxotroph (CC398 clonal type) requiring fatty acids for growth. Auxotrophy was confirmed by Exo profiles on both BHI-FA and BHI-FA-T. The minor *Staphylococcus* population (appearing as single large colonies in BHI-FA) is assigned as *Staphylococcus hominis*, which is reported to be a commensal and opportunist pathogen.



**FIG 5** Contribution of fatty acid (FA)-rich environments to diversity of *S. aureus* anti-FASII resistance. Intensive use of triclosan has led to its accumulation in numerous environments, including FA-rich animal biotopes. High-FA environments may select for diverse resistance mechanisms, including those that require exogenous fatty acids. They comprise triclosan-resistant *fabI* variants that were identified independently of FA (22, 23, 41–43); our results show that resistance frequencies are even greater in FA-rich environments. FA-rich environments favor emergence of FASII bypass mutants affected in initiation genes *fabD* and *accDA accBC* (6, 19, 29); the present work shows that FASII bypass is frequent among clinical isolates and correlates with genetic polymorphisms in *fabD* and *acc*. Fatty acid auxotrophs (nonculturable without fatty acids) and normally growing isolates constitute this novel clinical group of resistant staphylococci. Anti-FASII resistance under low-FA conditions leads to endogenous phospholipid (PL) membranes. FASII bypass under high-FA conditions leads to a flexible membrane composition that varies according to what fatty acids are available in each biotope.

FA<sup>T</sup> clinical and veterinary isolates that incorporate exogenous fatty acids comprise a common class of anti-FASII-resistant *S. aureus*. FASII bypass is attributed to polymorphisms in *acc* and/or *fabD* genes, including a *fabD* mutation previously identified as responsible for FASII bypass and resistance (19). Most isolates of this class were from superficial skin wounds in humans and mastitis cases in cows. Both infection sites are fatty acid rich (13, 26).

This study also uncovers a new class of unculturable staphylococci among clinical and veterinary isolates, which are anti-FASII resistant and require fatty acids for growth. A previous report suggested that fatty acid auxotrophs would be noninfectious based on the behavior of constructed *S. aureus acc* mutants in a mouse model (6). In contrast to those conclusions, this study establishes that at the least, fatty acid auxotrophs (i) are maintained in *in vivo* reservoirs and (ii) define a new category of VBNC pathogens that would escape detection in current routine identification screens. Other Gram-positive bacteria may also generate infectious fatty acid auxotrophs, as exemplified here by identification of *S. hominis*, a coagulase-negative species, and a *Corynebacterium* isolate closely related to benign *C. accolens* species, whose presence is a predictor of *S. aureus* nasal carriage (32, 33). To date, identification of fatty acid auxotrophs among clinical isolates is rare; recently, an *Enterococcus faecalis* auxotroph was isolated from an umbilical exudate (34), and an *S. aureus* strain mutated in the FabF fatty acid synthase was isolated from a patient with septic arthritis (35). A small-colony variant of *S. aureus* isolated *in vitro* was restored to normal growth by fatty acid supplementation, indicating a possible FASII defect (36). Taken together, these findings suggest that fatty acid auxotrophs, not systematically searched, may be common in the host. Our work suggests a straightforward means for their detection by including fatty acids in the screening medium.

We recently showed that *S. aureus* FASII bypass variants isolated in triclosan and fatty acid selections were cross-resistant to a second FabI inhibitor, AFN-1252 (renamed Debio 1452 [2, 3]); inversely, AFN-1252-resistant mutants were also triclosan resistant (19). A drug related to Debio 1452 is being tested in phase II clinical trials to treat skin infections (ClinicalTrials.gov registration no. NCT02426918). Based on our findings, AFN-1252 treatment would expectedly be ineffective in patients harboring FASII bypass isolates, including fatty acid auxotrophs.

In conclusion, the fatty-acid-dependent strategy of anti-FASII resistance is frequent and likely relevant to infection, and it may favor diverse mechanisms of resistance (Fig. 5). Emergence of resistance may be due to the presence of both triclosan (37) and fatty acids in numerous host biotopes (19). Two distinct resistance classes are discerned: (i) alterations in FabI, the triclosan target, lead to variants that produce membranes with endogenous membrane fatty acid composition, and (ii) alterations in alternative FASII genes, e.g., *fabD* and *accDA accBC*, lead to flexible membrane fatty acids whose composition varies according to the host biotope (Fig. 5). These results raise caveats on the use of FASII inhibitors for sustainable antimicrobial treatment and argue for an even more restrictive use of triclosan than advised in recent European and American legislations (see Commission implementing decision [EU] 2016/110, <https://www.federalregister.gov/articles/2016/09/06/2016-21337/safety-and-effectiveness-of-consumer-antiseptics-topical-antimicrobial-drug-products-for>).

## MATERIALS AND METHODS

**Ethics statement.** *S. aureus* isolates were from patients hospitalized from 2012 to 2014 in Percy Military Hospital (Clamart, France) and were provided anonymously after isolation. Additional *S. aureus* isolates were from previously published collections. Specimens (sputum, biopsy samples, skin samples, etc.) were collected during the usual care of patients, and the collections were approved by the hospital ethics committee.

**Bacterial isolates and handling.** A collection of 695 *S. aureus* isolates (629 human clinical and 66 cow mastitis isolates) was screened for triclosan resistance in the absence or presence of fatty acids (FA-T<sup>r</sup> phenotypes). Isolates were obtained from Percy Military Hospital (482 isolates), the Harmony collection (91 isolates) (38), and our laboratory collection (122 isolates). Isolates other than those from the Harmony collection were from French patients. The Harmony collection comprises methicillin-resistant *S. aureus* (MRSA) isolates representative of epidemic or otherwise common nosocomial clones collected between 1981 and 1998 from 11 European countries. We do not have information on exposure of patients or farm animals to triclosan. However, triclosan has had widespread use worldwide since the 1970s, making exposure likely in all individuals (18). Human isolates with specified sample origins (381) were from healthy carriers (42) and patients suffering from infections corresponding to superficial (177) and deep wound (14) exudates, surgical drains (10), bone biopsy samples (57), pulmonary samples (sputum, bronchial aspirate, or pleural fluids) (39), otorhinolaryngological samples (7), or blood cultures (35). *S. aureus* was isolated after purification on mannitol salt plates according to routine procedure.

To screen for isolates that grew only in the presence of fatty acids (i.e., fatty acid auxotrophs), primary samples (133) of diverse origins were collected from 103 patients (Percy Military Hospital; see Table S1 in the supplemental material). They were spread directly on fatty-acid-containing plates (see below). The same patient samples were analyzed by routine clinical tests for bacterial identification (mannitol salt agar [Chapman medium] for *S. aureus*).

**Screening for fatty-acid-dependent triclosan resistance.** Four types of agar plates were prepared to identify the triclosan resistance status of isolates: brain heart infusion (BHI), BHI-triclosan (BHI-T), BHI-fatty acids (BHI-FA), and BHI-fatty acids-triclosan (BHI-FA-T). Triclosan was used at 0.25  $\mu\text{g}/\text{ml}$ . BHI-FA and BHI-FA-T comprised an equimolar mix (170  $\mu\text{M}$ ) [each] fatty acids found in human skin (20) and serum (21): myristic acid (C14:0), palmitic acid (C16:0), and oleic acid (C18:1) (Sigma-Aldrich). Harmony and laboratory collection isolates were spotted directly (5  $\mu\text{l}$ ) from thawed culture stocks on the four types of medium. For isolates received on fresh slants from Percy Hospital, colonies were resuspended in 200  $\mu\text{l}$  of 0.9% NaCl before spotting. Plates were incubated at 37°C for 20 h and then photographed. Isolates that grew on BHI-FA-T but grew poorly or not at all on BHI-T were scored as fatty acid dependent and triclosan resistant (FA-T<sup>r</sup>) and studied further.

**Fatty acid auxotroph screening test.** To identify fatty acid auxotrophs, primary clinical samples were isolated on a modified HiChrome agar medium (HiMedia Laboratories, supplied by Sigma) supplemented with oleic acid (C18:1) (500  $\mu\text{M}$ ), which is not toxic for *S. aureus* growth (39). Egg lecithin, an optional additive to HiChrome medium, was omitted to control the fatty acid content (egg lecithin comprises phosphatidyl ethanolamine lipid). Tellurite was added as per the supplier's recommendation to distinguish staphylococci, which give dark to black colonies, from other bacteria. A second plate contained added triclosan (0.25  $\mu\text{g} \cdot \text{ml}^{-1}$ ) to confirm triclosan resistance in primary samples, as expected for fatty acid auxotrophs. After 48 h of incubation, colonies (both tellurite stained and cream colored) present in the plate deprived of triclosan were subjected to the four-plate screen as described above to identify fatty acid auxotrophs. Those that grew only on fatty-acid-supplemented medium (BHI-FA and



BHI-FA-T) were reisolated, stored, and tested for fatty acid profiles and *spa* typing (see Table S3 in the supplemental material).

**Fatty acid analysis.** Aerated 4-ml bacterial cultures were prepared in BHI, BHI-FA, or BHI-FA-T. Whole-cell fatty acid profiles were determined as described previously (40), except that gas-liquid chromatography was performed in a split-splitless injection mode on an Autosystem XL chromatograph (PerkinElmer) equipped with a ZB-Wax (J&W Scientific) column (30 m by 0.25 mm by 0.25  $\mu$ m; Phenomenex, France). Data were recorded and analyzed with a TotalChrom workstation (PerkinElmer). *S. aureus* fatty acid peaks were detected between 12 and 30 min of elution.

In some cases, fatty acid profiles shifted from exogenous to endogenous during strain reisolation and after storage. We classified clinical and veterinary isolates from fatty acid phenotypes determined in initial screenings, which reflected their primary potentialities.

***fabI*, *fabD*, *accBC*, and *accDA* sequencing.** Chromosomal DNA was extracted according to commonly used techniques. Briefly, *S. aureus* cell suspensions were lysed by treatment with 100  $\mu$ g  $\cdot$  ml<sup>-1</sup> lysostaphin (Sigma) for 1 h at 37°C, followed by use of standard DNA preparation methods and PCR conditions. Primers (Eurogentec, Seraing, Belgium) for *S. aureus fabI* gene amplification were 5'-GATAC AGAAAGGACTAAATCAAA-3' and 5'-TTCCATCAGTCCGATTATTATA-3'. In cases where these primers gave no product, we used primers 5'-TAGCCGTAAAGAGCTTGAA-3' and 5'-ATATTTTACCTGTAACGCCA-3' to amplify the central part of the gene. PCR amplification using primers 5'-TGCGAAGAAGTAGGCAATAT-3' and 5'-GCAACAATACTACCACGGT-3' was done to screen for an *S. haemolyticus fabI* (*sh-fabI*) copy as reported previously (22). Primers for *accBC* genes were either 5'-ACGGGTAGATGAAAACAAAC-3' and 5'-TCTTTTTCATCAGAGCAA-3' or 5'-AGTTGTCTGTAGTGACG-3' and 5'-CCAGTGATGCCTTCGACTTC-3'. For *accDA* genes, primers were either 5'-ATTGCTAGCATGGTTAAAGATTTTTTAATCGAAC-3' and 5'-GTGC GGCCGCTTCTATATAAGAACCAGATA-3' or 5'-AACATTCAACAGTCAAACGA-3' and 5'-AACTAATGATTGAATTGATGTAACG. Primers for *fabD* were 5'-GAAGTACTGTAGTTAAAGCACACG-3' and 5'-GCTTTGATTTCTTCGACT ACTGCTT-3'.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.02515-16>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.4 MB.

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